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(19)

(54) PRODUCING CHEMICAL PLANT METABOLITES BY SUSPENSION CULTURE

(71) We, AMERICAN CYANAMID COMPANY, a corporation organized and existing under the laws of the State of Maine, United States of America, of Berdan Avenue, Township of Wayne, State of New Jersey, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to the production of chemical substances by cultivating plant tissue under submerged, aerated conditions in a liquid culture. More particularly, this invention is concerned with the production of chemical plant metabolites.

It has been known for many years that plant tissue can be grown independently of the whole parent plants, by cultivating the tissues under conditions which are generally similar to those employed to cultivate microorganisms in the production of antibiotics. The art has recognized that such plant tissue culture potentially represents a source of valuable chemical plant metabolites, i.e. chemical substances which are produced during normal growth of the plants. However, this potential has remained unrealized despite the obvious advantage of a completely controlled growth environment which plant tissue culture offers, compared with conventional agricultural methods.

Thus, it has been proposed to produce valuable plant metabolites by cultivating whole root organs under submerged aerobic conditions in a suitable aqueous nutrient medium in a manner strictly analogous to that used in the production of antibiotics. Although this technique has achieved moderate success in the laboratory, it seems unlikely that it will be susceptible to commercial utilization, largely because of the mechanical problems of growing and handling a huge mass of coarse, fibrous root material. Another approach has involved growing undifferentiated single cells of the plant. Since even a single cell of a plant contains all the genetic information characteristic of the plant as a whole, it seems reasonable to expect that culturing single plant cells under suitable conditions ought to lead to the production of the metabolites normally associated with that plant. There have indeed been several published reports of the detection of normal plant metabolites in disperse, nominally undifferentiated cell growth systems. But, according to these reports, the metabolite concentration levels achieved have been well below those attained in normal plant development. Accordingly, such culturing of undifferentiated cells has found no practical application.

In accordance with the present invention there is provided a new process of producing a chemical plant metabolite by suspension culture. The process of this invention comprises the steps of (a) providing a first aqueous, aerobic nutrient medium having growing undifferentiated cells or cell clusters of a plant known to elaborate the metabolite submerged therein; (b) culturing said undifferentiated cells or cell clusters in a second aqueous, aerobic nutrient medium having a level of auxin-activity lower than that of said first medium whereby organ primordia of the plant are generated; and (c) cultivated the resulting organ primordia to produce the metabolite.

The process of the present invention may be carried out by inoculating a first aqueous nutrient medium with vigorously growing undifferentiated cells or cell clusters of the plant known to elaborate the metabolite. The growth of the undifferentiated cells or cell clusters is maintained in the medium for a period of time under submerged aerobic conditions in the presence of a substance providing auxin-activity in the medium. The undifferentiated cells or cell clusters are cultured in a second aqueous nutrient medium whose level of auxin-activity is lower than that of the first medium, whereby redifferentiation of the plant cells occurs with the appearance of plant organ primordia and with the con-

comitant elaboration of the metabolite. Growth of the redifferentiated plant cells in the second medium is maintained for a period of time under submerged aerobic conditions to produce a composition referred to as the "whole harvest mash". A "whole harvest mash" is the untreated composition obtained after cultivation of the organ primordia has proceeded to the point where the resulting accumulation of metabolite is optimized for all practical purposes. This growth results in the accumulation of the metabolites which may then be isolated and collected by conventional methods.

A "primordium" as used in this Specification is defined as a rudimentary organ identifiable as containing microscopic structural elements analogous to those seen in the corresponding organ of the normal plant embryo at some stage in its development. Thus, there may be root primordia which are recognizably similar to embryonic root tissue; cotyledonary primordia, similar to the embryonic stages of cotyledonary development; and stem and plumule primordia, again analogous to those parts of the normal embryo at some stage of its development. Under some circumstances, more complex primordia may appear, containing elements of more than one embryonic component, and indeed, under some circumstances, whole embryoidal structures can appear, these then being considered to be whole plant primordia.

It is essential to the process of the present invention that the undifferentiated cells or cell clusters should be further grown under conditions favorable to redifferentiation and the development of primordia. Thus, we have found that in those instances where cell differentiation has not occurred, or is prevented from occurring, there is no formation of normal chemical metabolites. These results contradict some of the published work on single cell cultures and, on the contrary, out own experiments lend support to the theory that the small degree of chemical metabolite formation observed in nominally undifferentiated cell systems by prior workers was probably due to the presence, albeit unrecognized, of differentiated centers in cell clusters of the systems. Be that as it may, we use growth conditions which result in a substantial degree of primordia formation, whereas the prior art has chosen growth conditions favorable to the maintenance of undifferentiated cells, i.e. deliberately avoided the formation of primordia.

As examples of the types of chemical plant metabolites that can be obtained by the process of this invention may be mentioned: alkaloids, such as strychnine and brucine from *Strychnos* species; the rauwolfia alkaloids from *Rauwolfia* or *Alstonia* species; vinblastine from *Catharanthus* species (formerly *Vinca* species); atropa alkaloids from members of the family *Solanaceae;* gums, from *Beta vulgaris* and *Malva* species; dyes from *Indigofera, Rhus, Beta,* and *Lawsonia* species; essential oils, from *Mentha, Lavandula,* and *Rosa* species; pesticides from *Chrysanthemum, Derris,* and *Lonchocarpus* species; resins, from *Pinus,* and *Juniperus* species; rubber, from *Parthenium argentatum, Heva, Ficus,* and *Taraxacum* species; flavoring agents, from *Sassafras, Smilax,* and *Betula* species; and cardiac glycosides, from *Convaleria, Digitalis.* Extensive listing of useful plant metabolites can be found in standard textbooks. However, the nature of the chemical plant metabolites obtainable by the present invention cannot be defined in terms of specific plant genera or families, still less in terms of actual products, since our work indicates that the invention is of general application to the entire seed-forming segment of the plant kingdom, that is, to the phylum *Spermatophyta* which consists of some 300,000 species.

In operating the submerged growth process of this invention, aqueous nutrient media are normally used which contain one or more metabolizable carbon sources such as carbohydrates or fatty acid derivatives, and an assimilable source of nitrogen such as nitrate ion, ammonium ion, glycine, or urea. The requirements of the various types of plant tissue vary somewhat in detail. However, materials such as glucose, sucrose, corn syrup, starch, dextrose, and other materials of this nature may be used as the preferred carbon source. In addition, the nutrient media will also normally contain mineral salts, including phosphates, chlorides, sulfates, and metals including sodium, potassium, calcium, magnesium and those elements needed in trace amounts. The trace metals are normally present as impurities in all but the most highly purified inorganic compounds. They may be added if purified chemicals are used in the media. The optimum mineral requirement of the particular culture used may be determined by routine experimentation. Mixtures of metallic salts, such as are used in media for mold fermentations, are often quite suitable. Another component normally present in the basic media, in addition to carbon source, nitrogen source and mineral salts, is vitamins. In general, at least one or more of the common vitamins may be required for optimum growth of the plant tissue by the process of this invention. These include thiamine, riboflavin, pantothenic acid and niacin. A mixture of these will assure satisfactory growth. A crude vitamin source, such as yeast extract, is particularly useful. As in the case of the mineral salts, the vitamin requirement of the particular tissue being used may be determined with relative ease. In general, a carbohydrate solution, fortified with minerals, and vitamins, is used for cultivation of the plant tissues. Suitable nutrient ranges in meq. per liter are potassium, 2 to

20; ammonium, 5 to 50; magnesium 0.2 to 2; calcium 0.5 to 5; phosphorus 0.25 to 2.5; and nitrate 0 to 50. Carbohydrates are suitably employed at a rate of from 5 to 50 grams per liter and preferably from 10 to 30 grams per liter. At that stage of the process when an auxin-active substance is present to promote the proper growth of an undifferentiated plant material it should be present in very dilute concentration in the aqueous medium. A level of from 1×10^{-4} to 0.5×10^{-7}, preferably about 5×10^{-6} molar is often suitable but somewhat higher or lower concentrations may be used.

The term "auxin", as used herein, is a general term for the growth promoting phytohormones or growth-regulating constituents of plants. Natural auxins, that is, those substances exhibiting growth regulating activity and occurring in living whole plants are generally considered to be either 3-indoleacetic acid or certain derivatives of this such as its esters and glycosides. Auxin-active substances include the natural auxins as well as chemical compounds of synthetic origin which possess growth promoting phytohormonal activity. Auxin-active substances are sometimes referred to as embryonic-type cell growth stimulants. Typical auxin-active substances suitable for use in the present invention are well known and can be exemplified by 3-indoleacetic acid, 3-indole-butyric acid, 1-naphthaleneacetic acid, p-chlorophenoxy-acetic acid, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid. Also known to the art are "anti-auxin" compounds, that is, compounds which can inhibit or neutralize the phytohormonal activity of the auxin-active substances. Typical anti-auxins known to the art are, for example, 1-naphthoxyacetic acid, cinnamic acid, tropic acid, 2,3,5-triiodobenzoic acid, and 2,4,6-trichlorophenoxyacetic acid.

Various aqueous nutrient media for the cultivation of undifferentiated plant cells have been described in the literature. For example, an adaptation of one medium taught in the literature consists of the following ingredients:

Component	Concentration mg./l.
Calcium nitrate	200
Magnesium sulfate	360
Potassium nitrate	80
Potassium chloride	65
Sodium sulfate	200
Sodium dihydrogen phosphate	16.5
Ferrous sulfate	2.5
Potassium iodide	0.75
Manganous sulfate	4.50
Zinc sulfate	1.50
Boric acid	1.50
Thiamine	0.1
Pyridoxin	0.1
Nicotinic acid	0.5
Glycine	3.0
Sucrose	20,000
Naphthaleneacetic acid	as required

Also useful is the known basal nutrient medium, of Heller, which contains:

Component	Concentration mg./l.
Potassium chloride	750
Sodium nitrate	600
Magnesium sulfate heptahydrate	250
Sodium dihydrogen phosphate hydrate	125
Calcium chloride dihydrate	75
Sucrose	20,000.0

plus micronutrients as follows:

Ferric chloride	1.0
Zinc sulfate	1.0
Boric acid	1.0
Manganous sulfate	0.1
Cupric sulfate	0.03
Aluminum chloride	0.03
Nickel chloride	0.03
Potassium iodide	0.01
Sodium molybdate	0.03

These media, as well as the several others also tabulated in the literature may be supplemented with auxin-active compounds or anti-auxins and/or cytokinens, as required, to achieve particular ends. Generally speaking however, the particular basic medium used is of no great consequence, serving only to optimize the growth results with a particular plant material. We have found the medium of Linsmaier and Skoog (see Example 29) or that of Gamborg (see Example 10) to be adequate as a starting point from which to develop optimal media for plant materials of all species.

To practice this invention, it is necessary first to develop a vigorously growing, undifferentiated cell culture of the desired species in an aqueous medium, and the state of the plant tissue culture art has now progressed to the point where this can be done at will by simple experimentation. For example, a single cell culture of *Catharanthus Roseus* may be obtained by inoculating germ free living stem segments of *Catharanthus roseus* onto the surface of an agar slant made up from a nutrient medium containing an auxin-active substance. Advantageously, there is added a cytokinen such as kinetin or zeatin as an auxiliary cell division factor. The nutrient medium contains the usual major mineral and trace elements, an assimilable source of carbon and of nitrogen such as ammonium or

nitrate ion and, preferably, one or more vitamins of the B family. After some days to weeks of incubation at 15°C. to 35°C., it is observed that "callus" tissue, which consists of a cluster of unorganized new cellular growth, has formed on the original inoculum segment. This new growth is excised and transferred aseptically to a fresh agar slant or, preferably, to liquid medium of the same composition (omitting the agar) and incubated on a shaker, again at 15°C. to 35°C. As further growth occurs, subdivision and additional transfers of the cell material to fresh medium are carried out, taking particular pains to transfer preferentially any smaller aggregates that have developed in the preceding incubation stage. After several such transfers to fresh medium, it is found that transformation of the material to a more disperse mode of growth has occurred, and, in fact, by a proper attention to details as is well understood by those skilled in the art, it is possible to arrive at a system consisting substantially entirely of small clusters of undifferentiated cells or single undifferentiated cells dispersed in an aqueous liquid medium. This is the ideal state for maintenance of the parental clone. Any selection or mutation of the plant organism can now be done on this material in exactly the manner that such operations have been carried out on microorganisms. Thus, selection of new cell lines (cell strains) from such a population, either with or without accompanying mutational procedures, can be done on the basis of cell morphology, pigment formation (or lack thereof), rate of growth, ability to grow on a selected medium, or indeed generally on the basis of any of the factors which have been classically used for the selection of industrial microbial cultures Most desirable, however, is selection based on the ability to produce an enhanced quantity of the desired metabolite in the productive, differentiated stage, and this selection is accomplished by trial.

The aqueous medium and other growth conditions required to cause the undifferentiated single plant cells or cell clusters to redifferentiate to the point of forming primordia and thus to enter the chemical metabolite-producing phase is again arrived at by experimentation. The undifferentiated cells and clusters, which can be designated "pre-embryos", are grown in a submerged aerated liquid system in exactly the same way as if they were micro-organisms, which in a sense, as free cells, they are. For this submerged aerobic culture, the medium is not solidified by a gelling agent, although low concentrations of thickeners such as agar or methyl cellulose can be useful in modifying the viscosity and therefore the state of shear in the stirred system. Otherwise, the growth medium again contains the usual major mineral and trace elemental ions, available carbon, available nitrogen, sulfur and phosphorus sources, and one or more B vitamins. The transition from the undifferentiated cell state to the redifferentiated or chemical metabolite producing-state is accomplished by culturing the undifferentiated material in a growth medium whose level of auxin-activity is lower than that of the initial growth medium. This diminished level of auxin-activity can be achieved, for example, by transfer of the undifferentiated cells to a fresh medium having a diminished auxin-activity level, or by dilution of the undifferentiated growth stage medium with fresh medium containing no auxin-activity, or by adding an anti-auxin compound to the undifferentiated growth stage medium after a satisfactory level of undifferentiated growth has been achieved. In the case where the auxin-active substance is metabolically unstable, as for example 3-indoleacetic acid, then the level of auxin-activity can be simply allowed to decay naturally, by means of extended incubation time. It is difficult or impossible to specify an absolute auxin-activity level at which undifferentiated growth will be maintained as this level is different for different auxin-active compounds and is also different for cells of different plant species. This interdependence of species and auxin-activity level for maintained undifferentiated growth is probably a consequence of varying sensitivity of different species to particular auxin-active compounds and may also depend on the level of endogenously produced auxin which also varies from one species to another. Therefore, the optimal level for a given auxin-active substance to maintain undifferentiated growth cells of a given plant species is determined by experiment. By such experimentation, using the most popular auxin-active substance, 2,4-dichlorophenoxyacetic acid, it has been found that about 2 to 20 $\times 10^{-6}$ moles per liter of this particular auxin-active compound is sufficient to maintain undifferentiated cell growth for many species of plant material. With too high a level of auxin-active substance, signs of toxicity such as poor growth or even cell death appear. At too low levels of auxin-active compound, the desired undifferentiated mode of growth is not maintained. More intensively active auxin-active compounds, such as 2,4,5-trichlorophenoxyacetic acid, must be used at lower levels than those mentioned above for 2,4-dichlorophenoxyacetic acid. Conversely, less active auxin-active substances, such as 1-naphthaleneacetic acid, must be used at somewhat higher levels than those mentioned for 2,4-dichlorophenoxyacetic acid.

Of course, when it is desired to bring about redifferentiation of a given plant material by culturing in a medium of reduced auxin-activity level, then it is convenient that the auxin-active compound level in the undifferentiated stage medium not be greatly higher than that required to just maintain growth in the

undifferentiated form. Thus, nly a minimal dilution with auxin-free medium is required to decrease the auxin-activity level to that which will permit redifferentiation to occur. Generally speaking, a dilution of at least two-fold with fresh auxin-free medium would be required to diminish the auxin-activity from the undifferentiated growth level to the re-differention-permitting level. Higher degrees of dilution would permit the use of less critical, higher, levels of auxin-activity in the undifferentiated stage. Of course, if the auxin-activity is removed by washing the undifferentiated cells, then no increase in final volume is required. This is also true (i.e., no volume increase is required) when addition of an anti-auxin is used to lower the effective level of auxin-activity.

Typically, for the induction of differentiated growth, the proper (lower auxin-activity) medium is inoculated with 5% to 50% of its volume of the disperse (undifferentiated) growth stage obtained as described for *Catharanthus roseus* above, and then incubated in a shaken, rotated, or stirred vessel at from 15°C. to 35°C., preferably 20°C. to 30°C., until organized differentiated growth has taken place to the desired extent. Under these conditions, the organized growth takes the form of small pellets, usually 0.5 to 4 mm. in their largest dimension, showing microscopic or even macroscopic structure corresponding to a structure seen in an organ or organs of the intact plant.

When a satisfactory growth level in the differentiated growth stage has been achieved, i.e., when substantial quantities of the desired metabolite have been formed, usually after from 3 days to 3 weeks, the slurry of plant primordia is harvested, and normally is then processed to recover the desired metabolite product. This can be accomplished, according to the nature of the metabolite product, by extraction with solvent, r by brief cooking followed by filtration to remove the spent plant solids, and specifically precipitating the desired product, or by separating the same by adsorption or ion exchange methods. The method of isolation generally will follow whatever process is used for isolating the given metabolite from whole plant material, and the present invention in no way resides in the specific method of isolation used.

In some instances, it may not be necessary to isolate the desired metabolite product from the mass of the plant primordia. Indeed, this mass may itself be the desired product; for example, the process of this invention can be applied to the high lysine strain of maize to result in the net conversion of cheap sucrose and ammonium or nitrate ion to high quality protein.

The invention is illustrated by the Examples which follow, wherein all parts and percentages are by weight unless otherwise indicated.

Example 1.

Induction of *Beta vulgaris* Callus Growth on Normal Plant Material and Maintenance on Solidified Medium

Seeds of common red beet, *Beta vulgaris* variety Detroit Select Globe, were germinated on moist filter paper, and when completely emerged, the germinated seedlings were sterilized by immersion in 1% sodium hypochlorite solution for 5 minutes. (After this point, all operations involving living tissue were carried out under conditions of rigorous asepsis). These seedlings were transferred onto a moist sterile filter paper circle and were cut up into the component parts of radical segments, hypocotyl segments, and cotyledon segments, and each of these segments was transferred to separate 4 inch petri plates containing 30 milliliters each of the following medium.

MS Medium.

	Component	Concentration mg./l.
The major mineral ions and nitrogen sources	Potassium nitrate	950
	Ammonium nitrate	720
	Magnesium sulfate heptahydrate	185
	Calcium chloride dihydrate	220
	Potassium phosphate.	68
Trace elements	Ferrous sulfate heptahydrate	30
	Manganous sulfate hydrate	7
	Zinc sulfate heptahydrate	4
	Boric acid, anhydrous	2
	Ammonium molybdate	0.1
	Potassium iodide	0.4
	Cupric sulfate pentahydrate	0.12
Carbon source	Sucrose	20,000
Solidifying agent	Agar	10,000
Vitamins	Biotin	0.1
	Choline chloride	1
	Inositol	1,000
	Nicotinic acid	1
	Pantothenic acid	1
	Pyridoxin	1
	Riboflavin	1
	Thiamin	1
Auxin-active compound	2,4-Dichlorophenoxyacetic acid	6
Cytokinin	Kinetin	0.06

Distilled water to make up one liter.

The medium was sterilized by atoclaving for 20 minutes at 15 psig steam pressure. The inoculated plates were incubated at 25°C. in subdued light and after three weeks, lumps of callus tissue were seen on the ends of several of the original inoculum pieces. A few plates were contaminated by fungi or bacterial overgrowth and these were discarded. The callus which appeared on the uncontaminated plates was transferred to 100 ml. flasks containing 30 ml. each of the same agar medium and the incubation continued for five weeks more under the same conditions. After this time sufficient growth had occurred that subculture to several further flask units was possible.

Example 2.

Induction of Unorganized Growth in Liquid Medium and Selection of Sub-Clones Having Desired Growth Properties

From each of several of the subcultured agar flasks of Example 1, an approximately eight millimeter diameter lump of dark brown callus tissue was transferred to a 250 milliliter Erlenmeyer flask containing 60 milliliters of medium of the composition indicated above, but omitting the agar. These flasks, now containing the inoculated liquid medium, were placed on a rotary shaker operated at 120 rpm with a shaking circle of 25 mm. and incubated with continuous shaking in diffuse light for four weeks at 25°C. At the end of this time, different results were seen in the various flasks, reflecting in some degree the inadvertent selection of cell types during the previous stages. Some flasks were seen to contain only one larger lump of callus, some contained the parental lump plus some smaller apparently indentical fragments representing sectors which had grown from the parent and which had been broken off during incubation, and some flasks contained broken off fragments which were themselves varied in appearance, probably representing further segregation of cell types.

Those flasks which contained smaller fragments and those which contained fragments of different appearance than the parental lumps were selected for further subculture and after six such stages of further selection, transfer and incubation, there was obtained a cell line which had the desired properties of growing rapidly in shaken flasks and growing in the form of completely unpigmented single cells and small aggregates, less than 0.5 mm. in diameter. These aggregates showed no fine structure under the microscope other than the component cells. The rate of growth was such that from less than 1% volume of cells in the inoculum, the cell volume after two weeks incubation on the rotary shaker was 20% of the total liquid volume. These properties remained stable upon mass transfer in the same medium.

Example 3.

Reinduction of Differential Growth as Plant Primordia in Submerged Aerated Liquid Culture

Th content of one of the flasks of disperse non-pigmented unorganized *Beta vulgaris* cell material as obtained in Example 2 was allowed to settle and the supernatant clear liquid was decanted off and discarded. The settled cells and cell clusters were washed twice by adding 60 ml. each time of auxin-free liquid medium (the medium of Example 1, omitting the 2,4-dichlorophenoxy-acetic acid and also the agar) and decanting the free liquid after allowing the cellular material to settle. The washed cells were then used in equal parts to inoculate ten 250 milliliter Erlenmeyer flasks, each containing 60 ml. of the auxin-free liquid medium, and these flasks were incubated on the rotary shaker as above for three weeks at 25°C. During this period of incubation, the inoculum cells and cell clusters multiplied extensively and toward the end of the period, redifferentiation occurred, to yield a slurry of bright orange and red particles which were seen under a low power microscope to consist almost entirely of tiny root primordia, each containing abundantly the normal pigment of the original cultivar of *Beta vulgaris* used.

Example 4.

Isolation of the Pigment

The slurry of root primordia from the ten flasks of the preceding Example was filtered, at which point all of the pigment was seen to be in the cellular fraction and none in the filtrate, which was discarded. The cell mass was reslurried in 500 ml. of water and the mixture boiled, resulting in the pigment being released to the liquid. Filtration resulted in spent cells, which were discarded, and a bright red filtrate which was further processed by passing it slowly through a one inch by 6 inch column of Dowex 50X2 ("Dowex" is a registered Trade Mark) cation exchange resin which retained essentially all of the pigment as a 2 inch bright red band near the top of the column. Elution of the pigment from the column was accomplished by the use of a 1% solution of sodium bicarbonate and the pigment was isolated from this by acidifying to pH 5, freeze drying the solution and extracting the free betanidine pigment from the freeze dried product with methanol. Betanidine is useful as a vegetable dye for food coloring purposes.

Example 5.

Larger Scale Growth of *Beta vulgaris* Primordia

Washed inoculum was prepared as in Example 2, and one flask of this was used to inoculate an intermediate stage consisting of 500 ml. of the liquid medium of Example 3

contained in a one liter borosilicate glass bottle. After 8 days shaking at 120 rpm at 25°C. the total contents of this bottle was used to inoculate ten liters of the same medium, contained in a one liter borosilicate glass carboy and this was then incubated using a magnetic stirrer for agitation and passing in sterile air at the rate of on liter per minute through a submerged sparger. The product was a red slurry of root primordia completely analogous to that obtained in the laboratory shaker flasks.

Example 6.

Derivation of Undifferentiated Cell cultures of *Digitalis purpurea* by Anther Culture

An unopened flower bud was removed from a field-grown plant of *Digitalis purpurea* and this was surface sterilized by brief immersion in 1% sodium hypochlorite solution. Under aseptic conditions the bud was dissected and the anthers were transferred to the agar surface of a petri plate which contained 15 ml. of the solidified MS medium as described in Example 1. After 18 days incubation at 28°C. several small callus clumps were seen growing from the anther explants. After an additional 20 days, extensive further growth of the undifferentiated callus clumps had occurred. The callus clusters were separated and transferred to fresh agar plates of the same medium and maintained on this medium by transfer every fourteen days.

Example 7.

Growth of *Digitalis purpurea* Undifferentiated Cells as Disperse Culture in Shaker Flasks

Callus clusters about 88 mm. in diameter from an agar plate of Example 6 were used as inoculum for each of five Erlenmeyer flasks containing 25 ml. of liquid sterile medium of the following composition.

MMS Medium

Component	Concentration, mg./l.
Potassium nitrate	950
Magnesium sulfate heptahydrate	185
Calcium chloride dihydrate	220
Potassium dihydrogen phosphate	68

Plus the trace elements, sucrose, vitamins, auxin-active compound, and cytokinen of the MS Medium.

After 18 days incubation the flasks showing the best and most disperse growth were combined, the cellular material was allowed to settle, the supernatant clear liquid poured off and the cell mass was washed with 50 ml. of fresh auxin-free MMSR medium of the following composition.

MMSR Medium

Component	Concentration mg./l.
Potassium nitrate	950
Magnesium sulfate heptahydrate	185
Calcium chloride dihydrate	220
Potassium dihydrogen phosphate	68
Ferrous sulfate heptahydrate	30
Manganous sulfate hydrate	7
Zinc sulfate heptahydrate	4
Boric acid	2
Ammonium molybdate	0.1
Potassium iodide	0.4
Cupric sulfate pentahydrate	0.12
Sucrose	20,000
Biotin	0.1
Choline chloride	1.0
Inositol (meso)	1,000
Nicotinic acid	1.0
Pantothenic acid	1.0
Pyridoxin	1.0
Riboflavin	1.0
Thiamin	1.0
Kinetin	0.06

The reconstituted cell suspension was shaken for five minutes and again allowed to settle and the clear supernatant poured off and discarded. The cell mass was washed once more in the same manner with MMSR medium.

Example 8.

Growth of *Digitalis purpurea* Cells Under Redifferentiating Conditions

The washed cell mass of the preceding example was used for inoculating twenty flasks (250 ml. Erlenmeyer flasks) each containing 25 ml. of MMSR medium. These flasks were incubated on the rotary shaker in subdued light at 28°C. for 10 days at which time each flask was seen to contain a multitude of small particles, each particle consisting of a small callus mass with one or several radiating primordial roots.

Example 9.

Detection of Steroidal Glycosides in Cultured Root Primordia of *Digitalis purpurea*

The flasks of *Digitalis purpurea* root primordia suspension of the preceding example were pooled and filtered by gravity through a fluted paper filter. The collected solid mass was dried at 60°C. overnight yielding fourteen grams of dry solid. One hundred milligrams of this product was refluxed with 3 ml. of 70% aqueous ethanol for an hour and the ethanolic extract separated by centrifugation. The ethanolic extract was evaporated in a stream of nitrogen to a volume of

0.9 ml. and the aqueous residue was extracted twice with chloroform. The entire chloroform extract was spotted on a silicic acid thin layer chromatographic plate and the chromatogram developed with a solvent mixture consisting of fifty parts cyclohexane, fifty parts acetone, and two parts glacial acetic acid. After development, the thin-layer plate was dried and sprayed with a saturated solution of vanillin in 50% aqueous phosphoric acid. The sprayed plate was heated in a hot air oven at 120°C. for fifteen minutes, at which time dark blue-gray spots were seen at Rf 0.32 and Rf 0.50, in exact alignment with control spots of digitoxin and digoxin on the same plate. Other steroid glycosides were seen at Rf 0.0, 0.05, and 0.80 but these were not positively identified.

Example 10.

Derivation of an Undifferentiated Cell Culture of *Datura stramonium*

Pot-grown seedlings of *Datura stramonium* were cut one centimeter above the soil level and the excised shoot was surface sterilized by immersion for one minute in 1% sodium hypochlorite solution. The whole shoot was then washed in sterile water and 5 mm. square portions of several leaves were explanted to the surface of agar G (Gamborg) medium in 100 mm. petri plates.

G Medium

Component	Concentration mg./l.
Potassium nitrate	2500
Ammonium sulfate	134
Sodium dihydrogen phosphate monohydrate	150
Magnesium sulfate heptahydrate	250
Calcium chloride dihydrate	150
Ferrous ethylenediaminetetraacetate	28
Manganous sulfate monohydrate	10
Boric acid	3
Zinc sulfate heptahydrate	2
Sodium molybdate dihydrate	0.250
Cupric sulfate pentahydrate	0.025
Cobaltous chloride hexahydrate	0.025
Potassium iodide	0.750
meso-Inositol	100
Nicotinic acid	1
Thiamine	10
Pyridoxin	1
Purified agar	1000
Sucrose	20,000
2,4-Dichlorophenoxyacetic acid	1
Kinetin	0.060

The pH is adjusted to 5.5 as necessary.

After five weeks incubation in the dark at 28°C. vigorous growth of callus clusters was seen on the surface and edges of the leaf explants. Several of these callus lumps were separated and each transferred aseptically to 250 ml. Erlenmeyer flasks containing 50 ml. of liquid G medium (composition as above but omitting the agar) and the flasks were incubated on the rotary shaker in subdued light at 25°C. As the undifferentiated callus material grew, it was subcultured to similar flasks on a 14-day cycle, selecting the more disperse material as it appeared, and after three such selections and transfers, a cell line was obtained which exhibited the desired mode of growth as single cells and small cell clusters.

Example 11.

Redifferentiation of *Datura stramonium* in Suspension Culture to Form Root Primordia

One flask of disperse undifferentiated cell culture of *Datura stramonium* as obtained in the above example was selected six days after the preceding transfer, and filtered aseptically by gravity on a 100 micron nylon mesh filter. The cell mass was washed with G medium lacking 2,4-dichlorophenoxyacetic acid and the resulting auxin-free cells used to inoculate a series of five flasks containing G medium but having respectively total concentrations of added 2,4-dichlorophenoxyacetic acid of 0.002, 0.004, 0.008, 0.016, and 0.032 mg. per liter. These flasks were incubated on the rotary shaker in subdued light at 28°C. for ten days, at which time the three flasks with the lowest levels of added auxin-activity showed copious formation of root primordia.

Example 12.

Isolation of Hyoscyamine and/or its Racemic Product, Atropine, from Cultured Differentiated Cells of *Datura stramonium*

One of the flasks from the preceding example (originally containing 0.004 mg. per liter of 2,4-dichlorophenoxyacetic acid) was filtered by gravity through a fluted paper and the collected mass of root primordia was washed with water and the resulting wet tissue mass (7 grams wet) was triturated with 5 ml. of 0.1 Normal aqueous sulfuric acid. The free liquid was separated by centrifugation, and this was made alkaline with concentrated ammonium hydroxide and extracted three times with 10 ml. portions of chloroform. The combined chloroform extracts was back extracted with 2 ml. of 0.1 Normal sulfuric acid to yield an aqueous concentrate of alkaloidal products, and this in turn was made alkaline with concentrated ammonium hydroxide and extracted with 0.6 ml. of chloroform. The chloroform extracted was evaporated to about 50 microliters and the entire quantity spotted at the origin of a silicic acid thin-

layer chromatographic plate. The plate was developed with a solvent mixture consisting of nine parts chloroform and one part diethylamine. After drying the developed plate, it was sprayed with iodoplatinate solution (0.5 ml. of 10% platinum chloride solution added to 25 ml. of 2% p tassium iodide solution) to reveal several alkaloid spots, the strongest falling at Rf 0.45, in exact alignment with a control spot of atropine on the same plate. The alkaloid usually found in the natural state in *Datura stramonium* plants is the optically active *l*-hyoscyamine but this material is ordinarily used in medicine in its racemized form which is known as atropine. Chromatography does not distinguish between the active and racemic forms.

Example 13.

Redifferentiation of *Datura stramonium* in Suspension Culture to Form Whole Plant Primordia

Sterile leaf explants of *Datura stramonium* were used as inoculum in shaker flasks of G-type medium containing 2 mg./l. of 2,4-dichlorophenoxyacetic acid. A callus formed within 8 days and this was transferred to fresh G-type medium containing 2 mg./l. of 2,4-dichlorophenoxyacetic acid and incubated an additional ten days to yield a disperse undifferentiated suspension of free cells and small cell clusters. This cellular material was washed with auxin-free G-type medium and used as inoculum for shaker flasks containing the auxin-free G-type medium. After 16 days incubation on the reciprocating shaker at 25°C., microscopic examination showed the presence of large numbers of whole plant primordia, that is embryo-like bodies corresponding in structure to the "heart-stage" and "torpedo-stage" of the normal plant embryo development.

Example 14.

Derivation of Undifferentiated Cell Cultures of *Rauwolfia serpentina*

Fresh commercial depulped fruits of *Rauwolfia serpentina* were shelled by carefully cracking the hard shell and removing the intact internal seed. These seeds were surface-sterilized by immersion for five minutes in 1% sodium hypochlorite and washed with sterile water. The sterile seeds were germinated by incubating on sterile moist filter paper in petri plates in an incubator at 28°C. Germination began in eight days and by about 18 days the seedlings had emerged completely from the seed coat. The sterile seedlings were transferred intact to the agar surface of petri plates containing MMS agar medium to which had been added by 10% by volume of sterile coconut milk. After two weeks, callus masses had appeared on the roots, stem, and cotyledons of the seedlings and this callus material was excised and transferred to fresh medium of the same kind in petri plates. Two further such transfers were made at two-weekly intervals and then selected portions of callus were transferred to 50 ml. portions of liquid MMS coconut milk medium in 250 ml. Erlenmeyer flasks. Disperse undifferentiated cell growth appeared in several of these flasks and this material was then maintained by bi-weekly transfer in G medium.

Example 15.

Growth of *Rauwolfia Serpentina* as Redifferentiated Material

Two flasks of the disperse growth of *Rauwolfia serpentina* from the preceding example were combined and the cell mass washed by decantation with three changes of G medium lacking the 2,4-dichlorophenoxyacetic acid. The auxin-free washed cell mass was used as inoculum for flasks of auxin-free G medium to which the following additions had been made:

Flask	Auxin-activity added
1	no addition
2	0.1 mg./l. of 1-naphthaleneacetic acid
3	1.0 mg./l. of 1-naphthaleneacetic acid
4	5.0 mg./l. of 1-naphthaleneacetic acid
5	1.0 mg./l. of 3-indoleacetic acid
6	5.0 mg./l. of 3-indoleacetic acid
7	10.0 mg./l. of 3-indoleacetic acid
8	20.0 mg./l. of 3-indoleacetic acid
9	0.5 mg./l. of 2,4-dichlorophenoxyacetic acid
10	1.0 mg./l. of 2,4-dichlorophenoxyacetic acid
11	5.0 mg./l. of 2,4-dichlorophenoxyacetic acid

Upon incubation on the rotary shaker for 8 days the first nine flasks (Nos. 1 through 9) showed varying degrees of redifferentiation (as evidenced by appearance of root primordia) ranging from slight in flask Nos. 5 and 9 to extensive in flask Nos. 1 and 8. These results are in accord with the generally recognized relative intensities of auxin-activity of the three substances employed, namely, that 2,4-dichlorophenoxyacetic acid is much more active than 1-naphthaleneacetic acid and this in turn is much more active than 3-indoleacetic acid. Thus, by transferring *Rauwolfia serpentina* cells from G medium containing 1 mg./l. of 2,4-dichlorophenoxyacetic acid to G-type medium containing no auxin-activity or to G-type medium containing 20 mg./l. of 3-indoleacetic acid, a net diminution in auxin-activity is accomplished and the desired redifferentiation occurs.